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### PLEYOTROPIC ANTIAGGREGANT EFFECTS OF AN INNOVATIVE ANTIARRHYTHMIC OF CLASS III SS-68, AN INDOLE DERIVATIVE

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#### Abstract

**Background:** A new indole derivative with the lab code-number SS-68 demonstrates a significant antiarrhythmic (anti-fibrillation) activity associated with the predominant influence on potassium and calcium conductivity of cardiomyocytes plasmalemmas. The preliminary data give evidence of SS-68 possessing an antiaggregant activity.

**Methods:** The influence of SS-68 on the platelet formation stage of hemostasis was determined by assessment of the anti-platelet effect (measuring platelet aggregation with Born/ O'Brien method), the dynamics of intracellular concentration of calcium ions in platelets was studied with FURA-2/AM fluorescent probe.

**Results:** Relative efficiency of the SS-68 indole derivative influence on platelet aggregation with the use of various inducers decreases in the following order: collagen > serotonin > ADP. The influence of SS-68 on platelet activity is characterized by pleiotropic effects: calcium-blocking effect, potentiation of cAMP-response, suppression of intracellular mechanisms of transmitting a stimulating signal, caused by the activation of collagen receptors.

**Conclusion:** G-proteins are the most likely molecular targets of pleiotropic antiaggregant effect of SS-68. A more than threefold increase in the anti-platelet effect of SS-68 in vivo suggests the development of an active metabolite (metabolites).

**Key words:** SS-68 compound; platelet aggregation; disaggregation; intracellular calcium; molecular targets of anti-platelet effect.

#### Background

In our previous research work, we discussed the possibility of the new indole derivative with the lab code-number SS-68, produced in the Research Institute of Physical and Organic Chemistry of Southern Federal University (Rostov-on Don), to demonstrate a significant antiarrhythmic (anti-fibrillation) activity associated with the predominant influence on potassium and calcium conductivity of cardiomyocytes plasmalemmas [1]. Besides, the SS-68 compound possesses a tropic effect on alpha- and beta-adrenoreceptors [2].

The aim of the present research work is to study

the influence of SS-68 on the platelet formation stage of hemostasis. For that purpose, we used a number of experimental conditions, modelling the platelets activation. The platelets reaction on external stimuli is determined by the correlation of extracellular signals, activating and inhibiting the cells activity [3]. The physiological stimulants of platelets activity may be divided into two groups: 1) weak agonists – ADP (in concentration < 1 mcM), vasopressin, adrenalin, serotonin; 2) strong agonists – thrombin, collagen, thromboxane A<sub>2</sub>, platelet activating factor (PAF), ADP (> mcM). The group of antagonists includes: prostaglandin I<sub>2</sub>, (prostacyclin, PGI<sub>2</sub>), prostaglandin

D<sub>2</sub> (PGD<sub>2</sub>), NO (nitrogen oxide, endothelial relaxing factor). They also may be classified according to their origin: plasma components (thrombin, plasmin, catecholamine), vascular (PGI<sub>2</sub>, NO, collagen), thrombocytic (ADP, serotonin, TxA<sub>2</sub>, PAF).

## Methods

1. Preparing suspension of washed platelets from whole blood.

1.1. Platelet-rich plasma (PRP) preparation. The blood (10 ml) was collected from the basilic vein into a plastic tube containing 1.5 ml of the additive (glucose – 5 mM; sodium citrate – 85 mM; citric acid (pH 6.55 – 65 mM). To obtain PRP, the blood was centrifuged at 200 g for 10 minutes, then 2.5 ml of the supernatant was carefully collected. The cell count was performed in the Goryaev chamber. To obtain plasma with platelet count of 250-350×10<sup>9</sup>/l, PRP was diluted with platelet-poor plasma.

1.2. Platelet-poor plasma preparation (PPP).

The supernatant, remained after PRP preparation, was centrifuged at 600 g for 20 minutes, then the transparent supernatant was collected from the tube.

1.3. The method of washed platelets preparation.

The method included sequential washing of a PRP sample in three modified Tyrode's solutions containing:

- T1 – 2.8 mM KCl, 2 mM MgCl<sub>2</sub>, 0.36 mM NaH<sub>2</sub>PO<sub>4</sub>, 138 mM NaCl, 0.2 mM EGTA, 10 mM HEPES;

- T2 – the composition is the same as in T1, but does not contain EGTA;

- T3 – 2.8 mM KCl, 0.5 mM MgCl<sub>2</sub>, 0.36 mM NaH<sub>2</sub>PO<sub>4</sub>, 138 mM NaCl, 10 mM HEPES, 1 mM CaCl<sub>2</sub>.

Before the investigation, the following substances were added to the solutions: to T1 and T2 – glucose to the concentration of 5 mM, bringing pH to 6.55; to T3 – pH 7.4.

In the process of washing platelets, the cell resuspension was performed in a slightly lower solution volume by gently shaking the tube for several minutes.

2. Measuring platelet aggregation with the Born/O'Brien method.

To assess the SS-68 influence on the induced change in light transmission, there were used the following aggregation parameters: 1) the maximum range – a percent increase of light transmission after adding an inducer; 2) the time between the start of aggregation and reaching the maximum of light transmission; 3) the maximum speed of aggregation.

The *in vitro* experiments were performed on rabbits weighing 2.5-3 kg, and *in vivo* – on male rats weighing 250-300 g. The blood for *in vitro*

experiments was collected from the rabbits' auricular vein, and *in vivo* experiments – from the rats' ventral aorta. The animals were anesthetized with chloral hydrate (400 mg/kg intraperitoneally) and were stabilized with 3.8% solution of sodium citrate at the ratio of 9:1. The SS-68 influence on intravascular aggregation of blood plates was measured in 2 hours after oral administration of the substance in the dose equimolar to 19 mg/kg of acetylsalicylic acid (the dose is equivalent to the ErC50 dose of acetylsalicylic acid obtained in *in vitro* experiments).

In the course of the investigation, 300 µl of platelet-rich plasma and the SS-68 solution in concentration of 1×10<sup>-4</sup>M (in *in vitro* experiments) were sequentially introduced into the aggregometer measuring cell. The samples were incubated in the thermostat of the aggregometer at 37°C for 5 minutes. After starting the recording of the aggregatogram, on the 10<sup>th</sup> second of the registration process, the aggregation inducer (adenosine 5'-phosphoric acid (ADP) in final concentration of 5 µM was added to the measuring cell. Acetylsalicylic acid was selected as a comparator agent.

3. The method of measuring intracellular calcium ion concentration in platelets with FURA-2/AM fluorescent probe was earlier described in [4, 5].

4. The statistical processing of the results.

The calculation of confidence intervals of experimental values and confidence assessment of their differences were performed with nonparametric Wilcoxon-Mann-Whitney paired test (in processing data of *in vivo* experiments) and Student t-test (in *in vitro* experiments), with the confidence level p=0.05.

## Results

1. Studying the SS-68 influence on platelet aggregation.

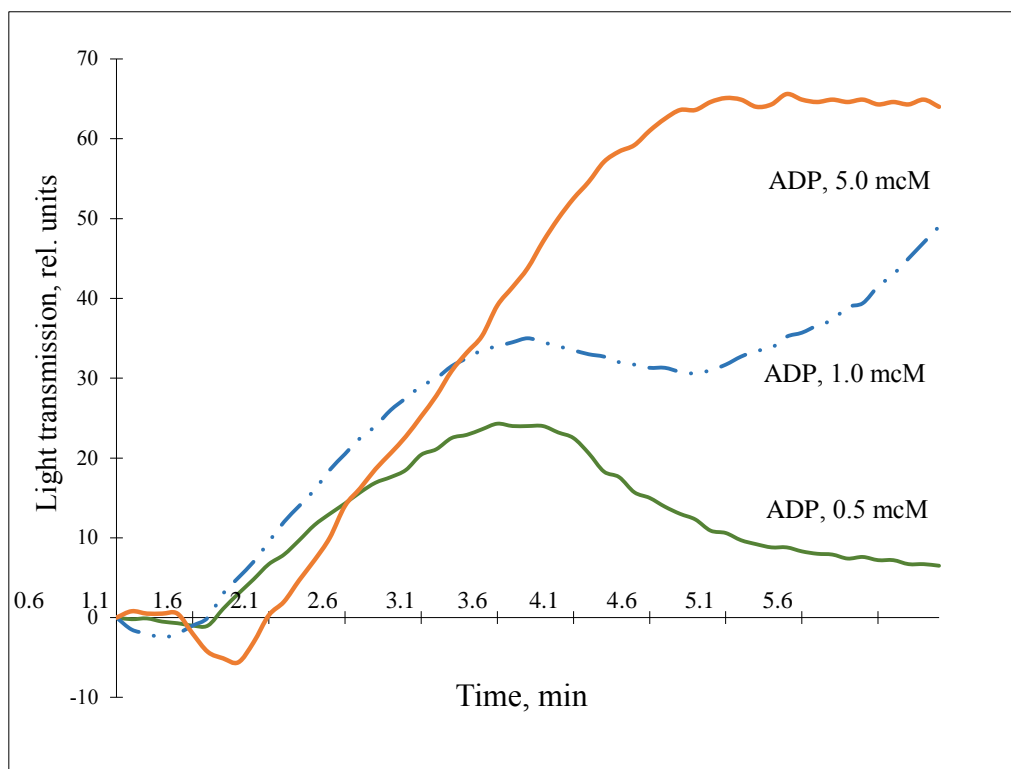
1.2. The SS-68 influence on platelet spontaneous aggregation. Any significant changes in the aggregation parameters when using SS-68 in the concentration range between 0.01-1.0 µM were not identified.

1.3. Studying the SS-68 influence on ADP-induced platelet aggregation. ADP is the most widespread inducer of blood plates used in *in vitro* experiments. According to the data published in the media, most researches on the drugs influence on the platelet function were conducted with the use of the above mentioned inducer [6]. This is explained by the fact that ADP is a universal endogenous regulator of platelets physiological activity, and it enables to separately register both reversible and irreversible phases of aggregation.

With low aggregate concentrations (below 5×10<sup>-7</sup>M ADP) there can be observed reversible aggregation, characterized by small aggregates (low-

amplitude fluctuations on the curve), containing 3-20 blood plates. Subsequent disaggregation manifests itself in a rapid decrease of the light transmission which does not reach the original level (Fig.1, the lower curve). With a higher dose ( $10^{-6}$  M ADP), there were registered the first and the second waves of

aggregation. And finally, the use of ADP in concentration of 5 mcM increased the transmission of light in the sample without its subsequent decrease, that characterizes irreversible aggregation (Fig. 1, the upper curve).



**Fig. 1.** ADP-induced platelet aggregation.

Note. The ordinate shows the change transmission of light, rel. units.

Conditions: the concentration of platelets in the sample is  $250-350 \times 10^9$  cells/l; Mixing speed – 800 rpm; Temperature –  $37^\circ\text{C}$

The study of the SS-68 influence on ADP-induced platelet aggregation (with ADP concentration of 1 mcM) has established, that in concentration of 0.01 and 0.03 mcM, SS-68 did not produce any optical density changes. Inducement of SS-68 in concentration of 0.1 and 0.5 mcM resulted in a significant decrease in the sphere-forming reaction and the appearance of 5-15 with a delay of aggregation initiation. The change in the other aggregation indicators was unstable: the decrease in the maximum amplitude of aggregation and its speed were of individual character, ranging 10-20%.

Apparently, the absence of significant changes in the reversible phase of aggregation with present SS-68 may be explained by particular qualities of the mechanism of ADP-induced aggregation of cells. The

ADP platelets induction (1 mcM and less) is known to be mediated by activation of  $P_2$ -purinoreceptors that are at the same time Ca-channels of the cell membrane [7]. Thus, the influence of ADP low concentrations on platelets is not mediated by formation of secondary messengers, and is not regulated by any known G-protein – the structures considered to be the target of the SS-68 activity [1, 2].

The results of the study of antiplatelet activity of SS-68 compared with antiplatelet activity of acetylsalicylic acid (ASA) are presented in Table 1 and Table 2. The conducted experiments have enabled to identify antiaggregant activity of SS-68. However, the platelet aggregation inhibition under the influence of SS-68 did not exceed 50% and gave way to the comparator agent – acetylsalicylic acid (Table 1).

Table 1

**Antiaggregant activity of SS-68 on the model of ADP-induced platelet aggregation in vitro**

Agent	$\Delta\%$ of inhibition			IC <sub>50</sub>
	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	
SS-68	42.1±1.1*	-	-	-
ASA <sup>1</sup>	52.06±4.2*	20.8±3.5*	4.6±1.7*	1.04·10 <sup>-4</sup>

Note: <sup>1</sup> Acetylsalicylic acid;

\* The figures are credible compared to control (Mann-Whitney test  $p < 0.005$ )

In *in vivo* experiments, the antiaggregant activity of SS-68 was three times as active as the comparator agent (Table 2).

Table 2

**Antiaggregant activity of SS-68 on the model of ADP-induced platelet aggregation in vivo**

Agent	Dose, mg/kg	$\Delta$ , % of inhibition
SS-68	35.8 <sup>1</sup>	93.6±1.6*
ASA	19	29.7±8.1*

Note: <sup>1</sup> Dose equimolar to the dose of acetylsalicylic acid;

\* The figures are credible compared to control (Mann-Whitney test  $p < 0.005$ )

1.4 The study of the SS-68 influence on serotonin-induced platelet aggregation.

According to current data, serotonin (5-hydroxy-tryptamine) belongs to weak inducers [8]. 5-HT<sub>2A</sub> type of serotonin receptors are present on the platelet cell membrane. It was shown, that in the process of binding serotonin with receptors, the activation of phosphoinositides exchange occurs, resulting in formation of secondary messengers – inositol-3-phosphate and diacylglycerol which mediate the cell response, primarily, at the expense of intracellular calcium mobilization. That is why we considered it best that we study the SS-68 influence on platelet aggregation induced by serotonin.

The data on the SS-68 influence on serotonin-induced platelet aggregation (serotonin was used in concentration of 10<sup>-5</sup> M) have shown, that in concentration of 0.1-1.0 mcM, SS-68 did not change any parameters of serotonin-induced platelet aggregation. The reliable difference from control values was observed when using SS-68 in high concentration of 5 mcM: the degree of maximum amplitude suppression ranged 10-15% compared to control; the time between aggregation initiation and achieving the amplitude maximum, as well as the maximum speed of aggregation were decreased by 11% and 9% respectively. The change in aggregation parameters had a positive correlation with the increase in concentration of SS-68 up to 15 mcM.

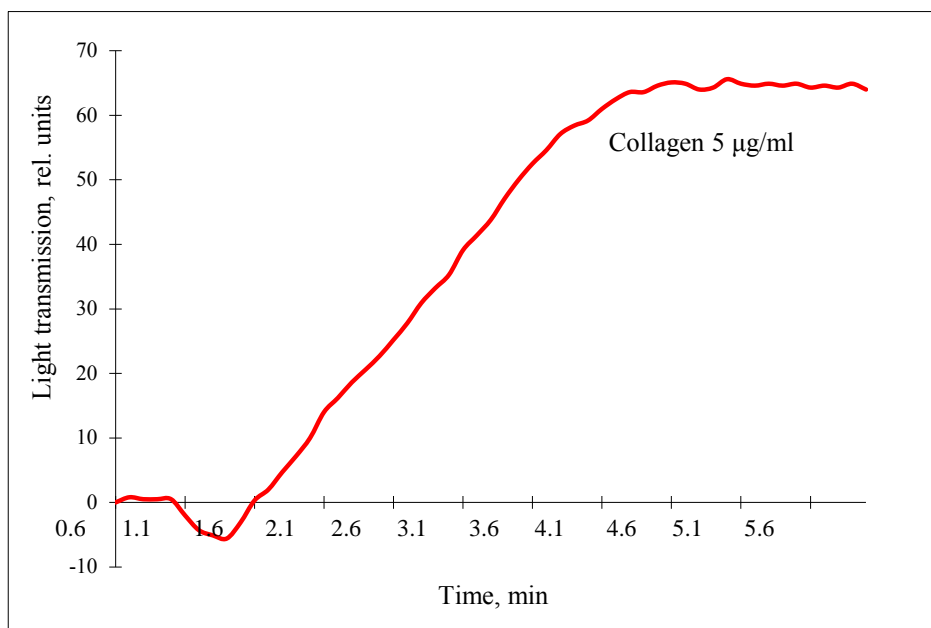
Thus, in serotonin-induced aggregation, SS-68 demonstrated a suppressive activity on aggregation in concentration of 5 mcM. Besides, there was noted some dependence of the effect on the concentration of SS-68 in the incubation medium.

So far, it is known that platelet activation by serotonin is regulated through cAMP-dependent pathways; it is blocked by AC-stimulators and PDE cAMP-inhibitors. The activity of adenylate cyclase biocatalysts is regulated through the functioning of the regulatory G-proteins, that can mediate a more pronounced influence of SS-68 on aggregation induced by serotonin.

1.5. The study of the SS-68 influence on collagen-induced platelet aggregation

The interaction of collagen with platelets receptors changes the form of cells and causes release reaction and irreversible aggregation. Collagen-induced activation of platelets includes the synthesis and release of thromboxane from cells – the activation factor of platelets, prostaglandins, ADP, and serotonin. The specified agents cause cells reaction through a receptor-mediated calcium entry from outside and ion exit from intracellular depots [9].

In this series of experiments, aggregation was induced with collagen in concentration of 5 mcg/ml. Collagen-induced aggregation is characterized by a latent period (10-15 c), it is monophasic and irreversible. A typical curve of changes in light transmission induced by collagen is shown in Figure 2.



**Fig. 2.** Collagen-induced change in light transmission of a platelet-rich plasma.

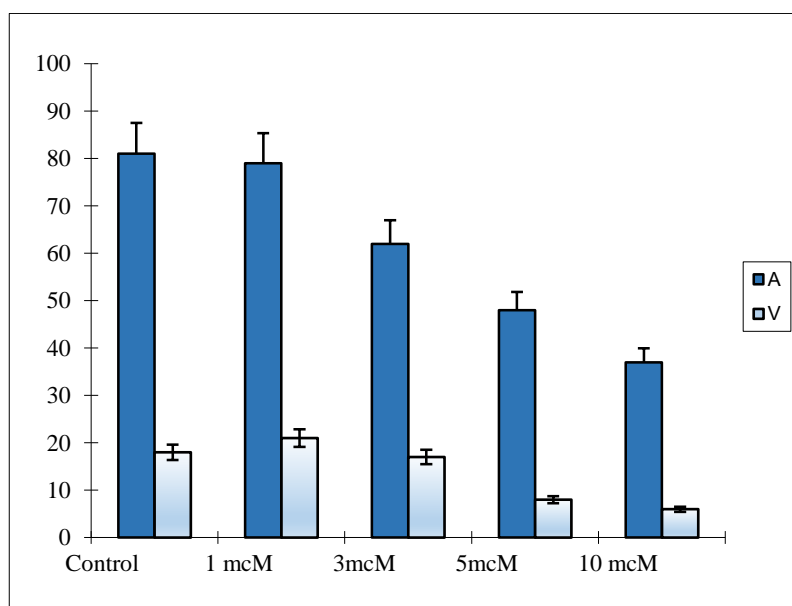
Note. Conditions: collagen – 5 mcg / ml;  $250 - 350 \times 10^9$  cells/liter; Mixing speed – 800 rpm; Temperature –  $37^\circ\text{C}$

The experiments revealed that the SS-68 effects coincide with the results of experiments, in which ADP and serotonin were used as inducers.

The results of this series of experiments demonstrate that the ability to decrease the platelets function in SS-68 on collagen-induced aggregation manifested itself to the most extent. Relative effectiveness of the SS-68 influence on platelet aggregation with the use of various inducers decreases in the following order: collagen

> serotonin >>ADP. That is why we have chosen a model of collagen-induced platelet aggregation for a detailed study of the mechanisms of the SS-68 activity.

Figure 3 demonstrates dependence of the light transmission speed (A) and maximum amplitude of aggregation (V) on concentration of SS-68. The analysis of the obtained data attracts attention to the dose dependence of the SS-68 effect.

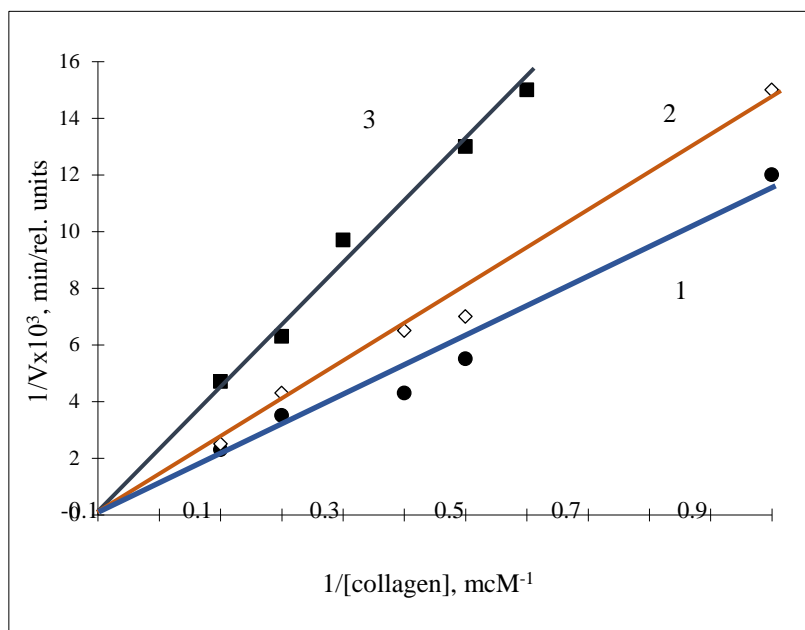


**Fig. 3.** Dependence of the light transmission speed (A) and maximum amplitude of aggregation (V) on concentration of SS-68



To determine the mechanism of the SS-68 activity, we have conducted an inhibitory analysis. The results of this analysis are shown in Figure 4, in the Lineweaver-Burk plot. The lower straight line presents the dependence of aggregation speed from inducer concentration; the upper straight lines characterize a joint effect of collagen and SS-68.

The straight lines meet at one point on the ordinate axis, that corresponds to the case of non-competitive inhibition. Therefore, SS-68 does not change receptors affinity to collagen, they decrease the number of “active” and functioning receptors. The increase in concentration of SS-68 from 1 to 10 mcM results in the increase of the effect.



**Fig. 4.** Dependence of the platelet aggregation rate on the inducer concentration in the absence of (1) and in the presence of SS-68 (2-1  $\mu$ M, 3-5  $\mu$ M), represented in the coordinates of Linuiver-Burke

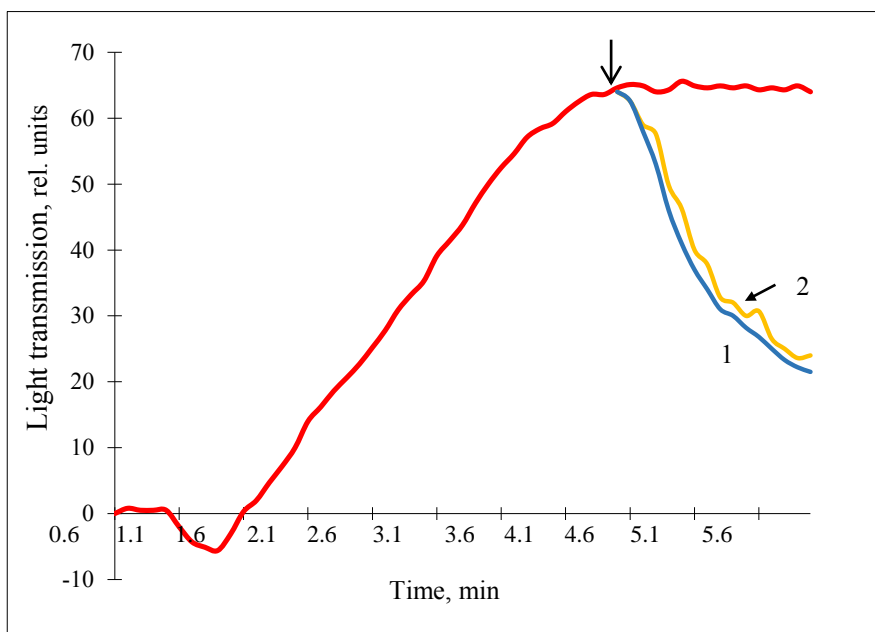
1.6 The study of the SS-68 influence on platelets disintegration.

The functional activity of platelets is determined by a correlation of factors of activation and inhibition [10]. The mechanism of suppressing SS-68 aggregation may be connected with activation of disintegration processes. This issue will be discussed in the second part of the research work.

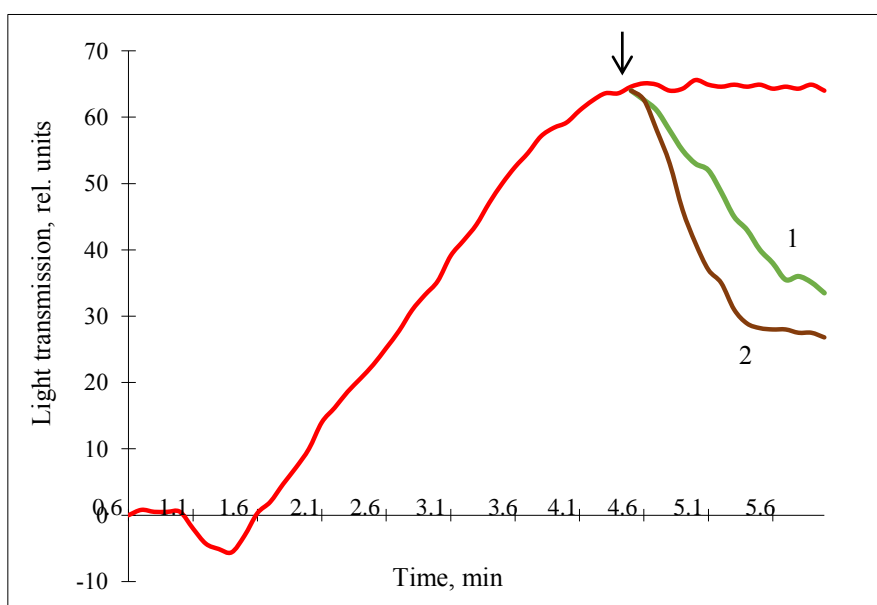
Disaggregation was induced with various inducers, different in their mechanism of activity, including nitroprussid sodium and adenosine. Nitroprusside sodium stimulates guanylate cyclase, providing the intracellular increase in cGMP [10]. The mechanism of adenosine activity is usually

connected with activation of AC and the increase in cAMP level in blood platelets.

Adding nitroprussid sodium (0.1 mcM) and adenosine (2.5 mcM) at the peak of aggregation caused platelets disaggregation (Figures 5, 6). The concentrations of inducers were selected in preliminary experiments with the condition they conform to the concentration of agents, when maximum amplitude of ADP-induced aggregation is 50% compared to control ( $IC_{50}$ ). In the study of the influence of hydrocortisone and prednisolone on disaggregation, the preparations were added to the sample together with nitroprussid sodium (or adenosine) at the peak of aggregation.



**Fig. 5.** The reaction of platelet disaggregation induced by sodium nitroprusside in the absence of (1) and in the presence of SS-68 (2). Note. The abscissa is the time, min; On the ordinate axis – change of light transmission, rel. units. Conditions: concentration of sodium nitroprusside – 0.1  $\mu$ M; SS-68 – 10  $\mu$ M; The number of platelets is  $250-350 \times 10^9$  cells/l; Mixing speed – 800 rpm; Temperature – 37°C



**Fig. 6.** Effect of SS-68 on platelet-induced disaggregation induced by adenosine.

Note. The abscissa is the time, min. On the ordinate axis – change of light transmission, rel. units. Conditions: Adenosine concentration 2.5  $\mu$ M; SS-68 – 10  $\mu$ M; The number of platelets is  $250-350 \times 10^9$  cells/l; Mixing speed – 800 rpm; Temperature – 37°C

It was found that SS-68 in concentrations of 1-10 mcM did not possess any disaggregating activity and did not change the platelets response to nitroprussid sodium. At the same time, SS-68 dose-dependently gave potential to adenosine-mediated disaggregation of platelets. For instance, with 1 mcM of SS-68,  $IC_{50}$  for adenosine was 1.7 mcM, and with the SS-68 concentration of 10 mcM, the  $IC_{50}$  value

was one order less than the original value (0.4 mcM).

2. The analysis of the SS-68 influence on the level of calcium ions in platelets.

The random level of  $Ca^{2+}$  in platelets, identified with FURA-2/AM fluorescent dye, was  $65 \pm 8$  nM. The study of the SS-68 influence on  $[Ca]_{ic}$  showed that in concentration of 1-5 mcM, SS-68 did not cause any changes in the random level of calcium ions.

Addition of washed collagen platelets (5 mcg/ml) to the suspension caused an increase in intracellular concentration of  $\text{Ca}^{2+}$  up to  $560 \pm 72$  nM. The growth of calcium level began right after adding the inducer, reached its maximum after 2-3 minutes, and then the fluorescence gradually declined within 10 minutes of observation to a new level which was significantly higher than the original one.

To ascertain whether calcium response of cells to collagen depends on intracellular ion concentration, we compared  $[\text{Ca}^{2+}]$  changes in platelets, induced with collagen, in the conventional medium containing 1 mM of  $\text{CaCl}_2$  and in calcium-free buffer (0.1 mM EGTA). It was found that in calcium-free buffer, the increase in  $\text{Ca}^{2+}$  was credibly lower ( $148 \pm 11$  nM). Therefore, the growth of  $\text{Ca}^{2+}$  level under the influence of collagen takes place mainly due to the entry of calcium ions from extracellular space.

To specify the mechanism of the SS-68 activity, we used a selective inhibitor  $\text{Ca}^{2+}$ -ATPase reticulum thapsigargin. On the background of thapsigargin activity (1 mM), causing emission of  $\text{Ca}^{2+}$  from intracellular depots and inhibiting its further accumulation, any credible influence of SS-68 on  $[\text{Ca}]_{\text{ic}}$  was not revealed.

The study of the SS-68 influence on  $[\text{Ca}]_{\text{ic}}$  enabled to state that the credible decrease in the random level of calcium was seen only when high concentrations (over 10 mM) of SS-68 were used.

SS-68 combinations in concentration of 0.5-5.0 mM dose-dependently suppressed the growth of  $[\text{Ca}]_{\text{ic}}$  caused by collagen (Table 3). The SS-68 effect (E) was assessed by measuring the change in the amplitude of fluorescence (with excitation wavelength 350 nm) and is expressed in the following formula:

$$E = (1 - \Delta F / \Delta F_k) \cdot 100\%$$

The increase in concentration of SS-68 in the incubation medium up to 5-25 mM resulted in more significant changes in the inducer-dependent growth of  $[\text{Ca}]_{\text{ic}}$ .

Table 3

### The SS-68 influence on stimulated calcium level in platelets

Concentration of SS-68, mM	$\Delta \text{Ca}^{2+}$ with SS-68 activity, nM
Control	$495 \pm 59$
0.5	$358 \pm 42^*$
1	$310 \pm 37^*$
5	$276 \pm 33^*$
10	$220 \pm 26^*$
25	$198 \pm 23^*$

**Symbols.**  $\Delta \text{Ca}^{2+}$  – difference between random and collagen-stimulated levels of  $\text{Ca}^{2+}$ .

\* The figures are credible compared to control (Student t-test  $p < 0.005$ ).

The study of time dependency of the  $\text{Ca}^{2+}$ -blocking effect of hydrocortisone on the collagen-induced growth of  $\text{Ca}^{2+}$  revealed the fact that the latent period for manifestation of the SS-68 calcium-blocking activity is not necessary (Table 4).

Table 4

### Changes in intracellular ion concentration of $\text{Ca}^{2+}$ induced in human blood platelets with collagen (5 mcg/ml) and SS-68 (1 mM)

Experimental conditions	$\Delta \text{Ca}^{2+}$ , nM
Collagen	$560 \pm 72$
SS-68	$28 \pm 13^*$
SS-68 + collagen	
time of pre-incubation:	
5 min	$323 \pm 27^{**}$
15 min	$344 \pm 31^{**}$
30 min	$315 \pm 29^{**}$

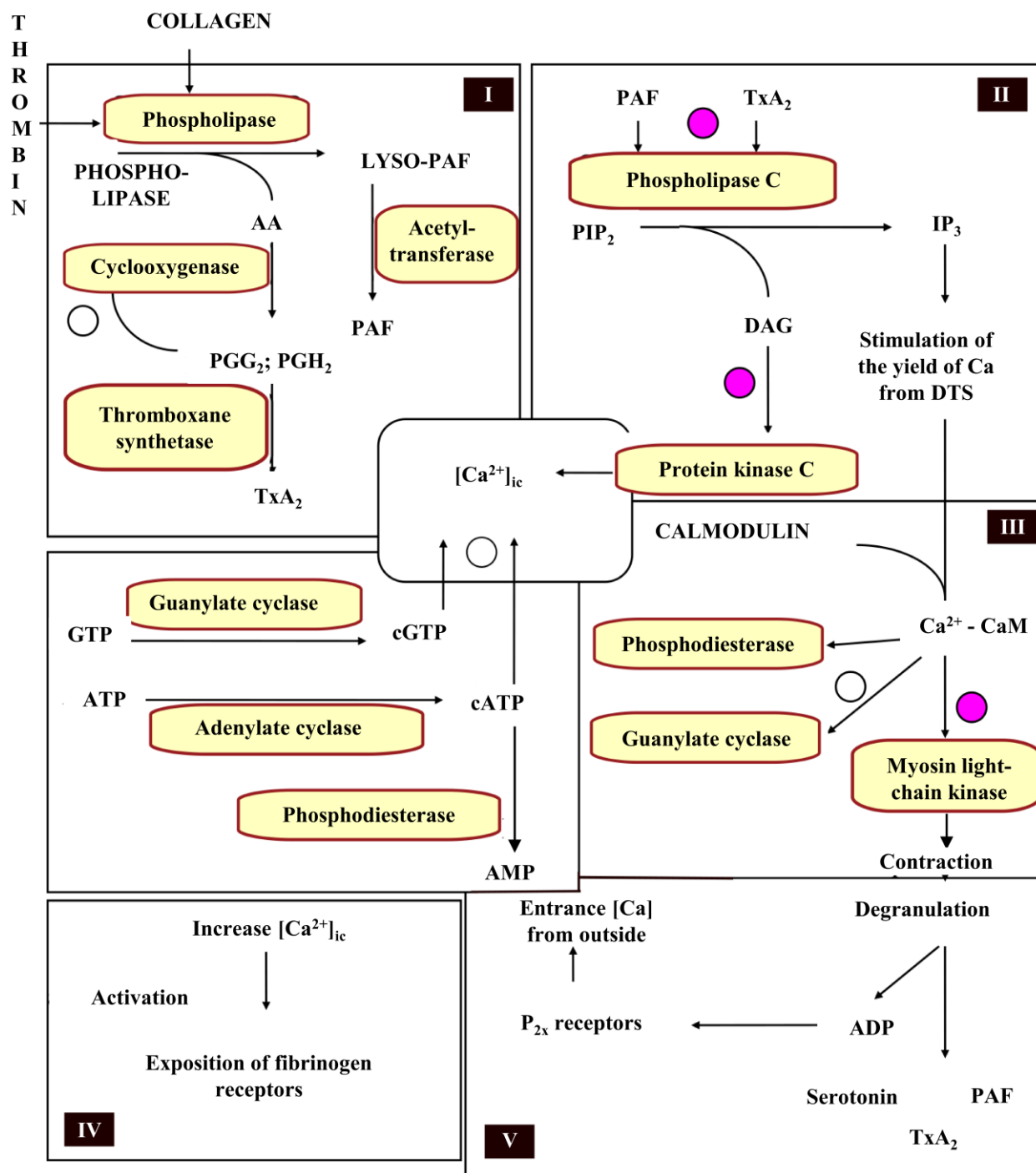
**Symbols.**  $\Delta \text{Ca}^{2+}$  – difference between random and collagen-stimulated levels of  $\text{Ca}^{2+}$ .

\*  $p < 0.05$  compared to samples containing only collagen;

\*\*  $p < 0.05$  compared to samples containing only SS-68.

The diagram in Figure 7 demonstrates a cascade mechanism of platelets activation and possible points of SS-68 application on collagen-induced platelet aggregation. The process of activation can be divided into 5 consecutive biochemical stages [11].





**Fig. 7.** Cascade mechanism of platelet activation and possible points of application of the action of compound SS-68 on collagen-induced platelet aggregation.

Note: ○ – stimulating effect; ● – inhibitory effect; Arachidonic acid (AA); Platelet activating factor (PAF); Thromboxane A<sub>2</sub> (TxA<sub>2</sub>); Phosphatidylinositol diphosphate (PIP<sub>2</sub>); 1,4,5-inositol triphosphate (IP<sub>3</sub>); Dense tubular system (DTS), Diacylglycerol (DAG); Guanosine triphosphate (GTP); Cyclic guanosine monophosphate (cGMP), Adenosine triphosphate (ATP); Adenosine diphosphate (ADP); Cyclic adenosine monophosphate (cAMP), Adenosine monophosphate (AMP); Calcium-Calmodulin (CaM)

I. Initiation of activation of the platelet stage of hemostasis is determined by formation of a complex of collagen with a specific membrane receptor of collagen [12]. This interaction results in activation of a membrane-bound enzyme of phospholipase A<sub>2</sub> with

formation of arachidonic acid (AA) and lyso-PAF. The reaction, catalyzed with an enzyme of thromboxane synthetase, produces thromboxane A<sub>2</sub> (TxA<sub>2</sub>) – a powerful aggregation inducer and vasoconstrictor. Another strong aggregation inducer – a platelet

activating factor (PAF) – is produced from an intermediate substance – lyso-PAF – through enzymatic acetylation with acetyltransferase.

Thus, two active inducers are produced during the first stage of platelets activation, and 1 molecule of collagen stimulates the synthesis of about 1000 molecules of PAF and TxA<sub>2</sub>.

II. The auto- and paracrine mechanism of platelets activation under the influence of PAF and TxA<sub>2</sub> is mediated by specific membrane receptors and is determined by stimulation of the activity of a membrane-bound enzyme of phospholipase C [13]. The enzymatic hydrolysis of a minor phospholipid of phosphatidylinositol diphosphate (PIP<sub>2</sub>) results in formation of two substances – 1,4,5-inositol triphosphate (IP<sub>3</sub>) and diacylglycerol (DAG), possessing the qualities of secondary intracellular mediators. IP<sub>3</sub> – is a hydrophilic chemical which mobilizes ionized calcium from its intracellular depots: a dense tubular system (DTS), and other structures of platelets endoplasmic reticulum. In resting platelets, the random level of calcium does not exceed 80 nM, thereat two pools of calcium are distinguished: 1) a cytosolic pool (t<sub>1/2</sub> ≈ 17 min) regulated by the Na/Ca transmembrane exchange; 2) a slowly changing pool (t<sub>1/2</sub> ≈ 300 min) depending on the activity of Ca/Mg-ATPase and localizing in DTS. The molecular target of DAG activity is the multifunctional membrane-bound enzyme of protein kinase C, performing phosphorylation of various intracellular proteins on serine and threonine residues. The regulatory role of PK C consists in activation of the intracellular mechanisms of decreasing the concentration of free ions of calcium in the platelet cytoplasm ([Ca<sup>2+</sup>]<sub>ic</sub>). These mechanisms include phosphorylation and subsequent inhibition of activity of receptor-operated Ca-channels of the cell membrane.

Thus, the key stage of stage II platelets activation is the mobilization of calcium ions from intracellular depots; ionized calcium is the main regulator of the sequential platelets activation [14].

III. The main intracellular processes under control of intracellular calcium level include: the positive modulation activity of membrane phospholipase A<sub>2</sub> and C; the cell surface expression of glycoprotein receptors of fibrinogen, integrin complexes (the exposition of some factors of adhesion is not a Ca-dependent process); the inhibition of adenylate cyclase and guanylate cyclase pathways slowing down the stimulation of platelets; the work of the platelet contractile apparatus which determines the change in the platelet form, adhesion activity, and secretion and release of the intracellular

granules content.

IV. The stage of degranulation. In the course of release reaction, through an open tubular system, platelets release adenosine diphosphate (ADP), PAF, vasopressin, serotonin, adrenalin, and TxA<sub>2</sub>, involving other platelets in the process of aggregation.

V. The final stages of platelets activation are associated with platelets surface expression of fibrinogen receptors – a glycoprotein complex of IIb/IIa [15].

**Conflicts of Interest:** The authors have no conflict of interest to declare.

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**Dukhanin Aleksandr Sergeevich**, Holder of Habilitation Degree in Medicine, Professor, Department of Molecular Pharmacology and Radiobiology, e-mail: [das03@rambler.ru](mailto:das03@rambler.ru). Determined by assessment of the anti-platelet effect of SS-68 (measuring platelet aggregation with Born/ O'Brien method), the dynamics of intracellular concentration of calcium ions in platelets was studied with FURA-2/AM fluorescent probe.

**Kucheryavenko Aida Fatihovna**, Holder of Habilitation Degree in Medicine, Associate Professor, Department of Pharmacology, e-mail: [aidakucheryavenko@yandex.ru](mailto:aidakucheryavenko@yandex.ru). In vitro experiments on rabbits and in vivo in rats.

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